I κ B α -mediated inhibition of v-Rel DNA binding requires direct interaction with the RXXRXXXC Rel/ κ B DNA-binding motif

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ABSTRACT Rel family proteins bind to κ B DNA sites, form heterodimers with one another, and modulate expression of genes linked to κ B motifs. I κ B factors associate with Rel proteins, inhibit Rel DNA binding *in vitro*, and displace DNA from DNA-bound Rel complexes. We have investigated the mechanism by which the p40/I κ B α inhibitor interferes with Rel DNA-binding activity. Here, we report that p40 contacts the RXXRXXXC DNA-binding motif conserved in all Rel family proteins, in addition to associating with the nuclear localizing sequence. Competition assays with a Rel-derived peptide comprising the DNA-binding region specifically alleviated p40-mediated inhibition of v-Rel DNA-binding activity, whereas a covalently modified Rel peptide was inactive. Combined, these results indicate that I κ B α interaction with the RXXRXXXC motif is required for inhibition of v-Rel DNA binding and suggest that nuclear I κ B factors may be critical for regulating transcription by Rel family proteins.

The v-Rel oncoprotein of avian reticuloendotheliosis virus strain T; its cellular homolog c-Rel, the *Drosophila* morphogen dorsal, and several NF- κ B-related proteins belong to the Rel family of transcription factors (1–7). Members of this family bind to κ B DNA sites, form heterodimers with one another, and modulate expression of genes linked to κ B DNA motifs. Sequences responsible for Rel DNA binding, dimerization, and nuclear localization map within the conserved N-terminal Rel homology region. Their divergent C-terminal ends participate in transcriptional activation (c-Rel, RelA, RelB) and in the regulation of Rel protein transport and DNA-binding activity (p52/100, p50/105; reviewed in ref. 1).

Inhibitors of NF- κ B function (I κ Bs) have been implicated in the regulation of Rel proteins both in the nucleus and in the cytoplasm. Cytoplasmic I κ Bs prevent the nuclear transport of Rel proteins by forming inactive I κ B–Rel complexes. Activation of these complexes involves the posttranslational modification and rapid degradation of I κ B, accompanied by the nuclear translocation and binding of active Rel proteins to κ B DNA sites (reviewed in ref. 1; see also refs. 8–16). More recently, I κ B factors have also been implicated in the regulation of nuclear Rel proteins. I κ B factors have been shown to enter the nucleus, to inhibit Rel DNA binding *in vitro*, and to dissociate NF- κ B protein–DNA complexes (17–21). This suggests that I κ B proteins may control the transcriptional activity of Rel and NF- κ B factors by removing nuclear Rel family proteins from κ B DNA-binding sites.

The association of I κ B factors with Rel proteins and the consequent inhibition of Rel DNA binding requires the ankyrin repeats common to all I κ Bs and to the cell cycle control cdc10, SWI4, and SWI6 proteins from yeast among others (reviewed in ref. 1), as well as the C-terminal region of some I κ B factors (22). This association was recently shown to also require the nuclear localizing sequence (NLS) of Rel

proteins (8, 23). Little is known, however, of the mechanism by which this interaction inhibits Rel DNA-binding activity. Here we report that in addition to its interaction with the NLS, p40/I κ B α directly contacts the RXXRXXXC v-Rel DNA-binding region conserved in all Rel family proteins. Our results indicate that this interaction is essential for p40-mediated inhibition of v-Rel DNA-binding activity and suggest that I κ B α may control the transcriptional activity of Rel family proteins by removing them from κ B DNA-binding sites in the nucleus (17–19).

MATERIALS AND METHODS

Plasmids. v-Rel mutants used in this study have been described (24). The deletion in dBamHI removes 210 amino acids from the C terminus of v-Rel. Mutants 2716 and 2726 carry point mutations that inactivate the NLS of v-Rel. db10 has an in-frame deletion of codons 2–48 in v-Rel. Mutant 2722 carries a cysteine to serine substitution at position 35 in v-Rel. Mutants 2721 and 3069 contain point mutations of conserved basic residues in the DNA-binding region of v-Rel. c-Rel and p40 proteins were expressed *in vivo* under the control of a spleen necrosis virus (SNV) long terminal repeat promoter. pIL6CAT expresses the chloramphenicol acetyltransferase (CAT) gene from the interleukin 6 enhancer/promoter (25).

Coprecipitation Assays. Wild-type and mutant v-Rel proteins were assayed for their ability to coprecipitate with chicken p40/I κ B α . Wild-type or mutant v-rel RNAs (0.5 μ g) were cotranslated in wheat germ extracts along with 1 μ g of p40 RNA and [³⁵S]methionine. Translation products were immunoprecipitated with anti-v-Rel antibodies raised against the unique C terminus of v-Rel (Ab1691; ref. 24) or with antibodies that recognize the unique envelope-derived N terminus of v-Rel (Ab1967; ref. 35).

The effect of excess cold κ B oligonucleotides on the association of p40 with v-Rel or v-Rel mutant 2722 was examined in coimmunoprecipitation assays. Cotranslated Rel–p40 complexes produced in rabbit reticulocyte lysates were incubated for 15 min at room temperature with or without 50 fmol of a double-stranded 27-mer κ B oligonucleotide containing a palindromic κ B-binding motif, 5'-CAACGGCAGGGGAATTCCCCTCTCCTT-3' (κ B-pd; ref. 26), prior to immunoprecipitation with anti-p40 antibodies (21). Proteins were resolved on SDS/10% polyacrylamide gels.

DNA-Binding Assays. Wild-type and mutant v-rel and chicken p40 genes were transcribed from SP6 or T7 promoters of pGem-derived expression vectors (Promega). *In vitro* translation reactions were performed in rabbit reticulocyte lysates (Amersham) with [³⁵S]methionine and 0.5 μ g of

rel-derived RNAs, alone or together with 1 μ g of p40 RNA. Aliquots (5 μ l) of the translation reaction mixtures were incubated with 50 fmol of the 27-mer double-stranded 32 P and BrdUrd double-labeled κ B-pd oligonucleotide (26) for 15 min at room temperature. After UV cross-linking, reaction mixtures were immunoprecipitated with anti-peptide antibodies against the unique C-terminal envelope-derived sequences of v-Rel (Ab1691) or with anti-p40 antibodies.

Cell Transfection and CAT Assays. COS-7 simian virus 40-transformed African green monkey kidney cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells (3×10^5) were cotransfected by a modified calcium phosphate procedure (27) with 5 μ g of SNV-c-Rel expression vector (34) or the c-Rel mutant 2722, 5 μ g of SNV-p40 or control vector DNA, 2 μ g of pIL6CAT (25), and 3 μ g of calf thymus DNA. CAT assays were performed 48 hr posttransfection (24).

Peptide Competition Assays. Peptide 1737 corresponds to residues 22–33 of v-Rel (EQPRQRGTRFRY; Multiple Peptide Systems, San Diego), which include the basic residues of the RXXRXXXC DNA-binding motif; peptide 1801 is derived from the unique C terminus of chicken c-Rel (residues 571–581) and contains an additional N-terminal cysteine residue for conjugation (CFYDTDGVHTDE). Peptides were added to DNA-binding reaction mixtures containing v-Rel or v-Rel plus p40 and incubated overnight at 4°C with gentle shaking. The following day, samples were further incubated for 15 min at room temperature, subjected to UV cross-linking, and immunoprecipitated with anti-v-Rel antibody Ab1691. Proteins were resolved on SDS/10% polyacrylamide gels.

Peptide Modification. Concentrated stocks of competing peptides 1737 and 1801 were treated with 10 mM *p*-hydroxyphenylglyoxal (pH 7.1) (HPG; Pierce) for 60 min at room temperature, before dilution and addition to binding reaction mixtures, as described (28, 29).

RESULTS

Mutations in the DNA-Binding and NLS Regions of v-Rel Impair Its Association with p40/I κ B α . The effects of I κ B factors on DNA-binding and subcellular localization of Rel proteins suggest that sequences involved in Rel protein–DNA contact and/or nuclear transport may constitute interaction sites for I κ B inhibitors. To address this issue, we analyzed the ability of mutant v-Rel proteins (Fig. 1A) to associate with the chicken inhibitor protein p40/I κ B α . Whereas 35 S-labeled p40 coprecipitated with wild-type v-Rel produced in cotranslation reactions (Fig. 1B, lane 1), Rel proteins with deletions (lane 2) or point mutations (lanes 3 and 4) in the NLS failed to show any p40 association, despite the presence of similar amounts of p40 in the cotranslation reactions (Lower). This result implicates the NLS region of v-Rel in a direct association with p40.

Interestingly, deletion of residues 2–48 in v-Rel also abolished interaction with p40 (Fig. 1B, lane 5). As this deletion removed residues essential for Rel/DNA contact (24), we examined the ability of v-Rel point mutants within this region to associate with p40 (Fig. 1A). Mutants with substitutions in the RXXRXXXC DNA-binding motif showed decreased association with p40, despite the fact that they harbored an intact NLS (Fig. 1B, lanes 7 and 8).

The results described above suggested that p40 may also contact the RXXRXXXC DNA-binding motif of v-Rel and prompted us to investigate the mechanism by which p40 affects v-Rel DNA binding. v-Rel or NLS mutants were cotranslated with chicken p40 and assayed for binding to oligonucleotides containing a palindromic interleukin 2 α receptor NF- κ B DNA motif (κ B-pd; refs. 24 and 26). p40 nearly abolished the DNA-binding activity of v-Rel but had no effect

FIG. 1. (A) Structure of wild-type and mutant v-Rel proteins. db10 lacks 210 amino acids from the C terminus of v-Rel. Mutations in 2716 and 2726 inactivate the NLS. v-Rel codons 2–48 were deleted in db10. Residues mutated in the DNA-binding region of 2721, 3069, and 2722 are indicated. (B) Coimmunoprecipitation of p40 with v-Rel mutants. (Upper) 35 S-labeled proteins were immunoprecipitated with Ab1691 (lanes 1 and 3–8) or Ab1967 (lane 2). (Lower) Equivalent amounts of p40 were present in nonimmunoprecipitated reactions.

on the binding activity of NLS mutants despite the fact that equivalent amounts of p40 were produced in all reactions (data not shown). This demonstrated that p40 association with the NLS region of v-Rel is required to inhibit Rel DNA-binding activity. In agreement with the results of Beg *et al.* (8) indicating that the C terminus of Rel proteins is not required for DNA-binding inhibition by I κ B α , p40 efficiently inhibited the *in vitro* DNA-binding activities of v- and c-Rel mutants with 138 and 276 amino acids, respectively, deleted from their C termini (data not shown).

Anti-Peptide Antibodies Raised Against the Rel DNA-Binding Motif Fail to Immunoprecipitate v-Rel Complexed with p40. The dramatic inhibition of v-Rel DNA binding upon association of p40 with the NLS region may result from p40 masking the v-Rel DNA-binding region and/or from a p40-induced conformational change in v-Rel that is incompatible with DNA binding. The fact that mutations in the RXXRXXXC DNA-binding motif of v-Rel consistently decreased p40 association (Fig. 1B) suggested that p40 may contact the DNA-binding region of v-Rel.

Immunoprecipitation assays using anti-peptide antibodies directed against the DNA-binding region (Ab1737) or the unique envelope-derived C terminus of v-Rel (Ab1691) provided further support for participation of the DNA-binding region of v-Rel in contact with p40. Unlike Ab1691, Ab1737 failed to immunoprecipitate p40 in complex with v-Rel (Fig. 2, compare lanes 2 and 5). Moreover, Ab1737 could only recognize free v-Rel, in contrast to Ab1691, which recognized

FIG. 2. Immunoprecipitation of v-Rel-p40 complexes with v-Rel anti-peptide antibodies. 35 S-labeled v-Rel produced alone in wheat germ lysates (lanes 1 and 4) or by cotranslation with p40 (lanes 2 and 5) was immunoprecipitated with anti-peptide antibodies directed against the DNA-binding region (Ab1737; lanes 1 and 2) or the unique envelope-derived C terminus of v-Rel (Ab1691; lanes 4 and 5). As a control, 35 S-labeled p40 was subjected to immunoprecipitation with Ab1737 (lane 3) and Ab1691 (lane 6).

both free v-Rel and v-Rel complexed with p40 (compare lanes 1 and 2 vs. lanes 4 and 5). Combined, these results demonstrate that the DNA-binding region of v-Rel is masked in p40-v-Rel complexes and suggest that the RXXRXXXC region may be in direct contact with p40.

Serine Substitution of Cys-35 in v-Rel Decreases p40-Mediated Inhibition of Its DNA-Binding Activity. To further address the role that the RXXRXXXC motif plays in p40-mediated inhibition of v-Rel DNA binding, we examined the effect of p40 on the binding activity of the (Cys-35 $\rightarrow$ Ser) v-Rel mutant 2722. In contrast to v-Rel, whose DNA-binding activity was greatly inhibited by p40 (Fig. 3A, lanes 1 and 2), the binding activity of mutant 2722 was never extensively inhibited by p40 (lanes 4 and 5). This suggests that DNA may not be excluded from p40-2722 complexes as efficiently as from p40-v-Rel complexes or that the on/off rate of p40 is different in p40-2722 and p40-v-Rel complexes.

Interestingly, immunoprecipitations with anti-p40 antibodies showed no DNA-bound v-Rel complexed with p40, whereas some DNA-bound mutant 2722 was detected (Fig. 3A, lanes 3 and 6). Furthermore, cotransfection assays in COS-7 cells showed that p40 inhibits activation of κ B site-dependent transcription by a chicken c-Rel protein containing the 2722 mutation 3- to 4-fold less efficiently than activation by wild-type c-Rel (Fig. 3C). Combined, these experiments show that p40 is less inhibitory to the DNA-binding activity of 2722 than to that of v-Rel.

Excess κ B Oligonucleotides Displace p40 from 2722-p40 Complexes. We next examined the effects of excess unlabeled κ B oligonucleotides on the stability of 35 S-labeled v-Rel-p40 and 2722-p40 complexes in immunoprecipitations with anti-p40 antibodies. Incubation with excess κ B DNA had no effect on v-Rel-p40 complexes (Fig. 3B, lanes 1 and 2). In contrast, excess κ B oligonucleotides decreased the amount of 2722 complexed with p40 by 59% (lanes 3 and 4). The displacement of 2722 from 2722-p40 complexes was dependent on κ B DNA sites, as excess oligonucleotides containing the non- κ B-related oct-1-binding site failed to reduce the amount of 2722-p40 complexes (data not shown). Our previous studies had shown that the cysteine residue mutated in 2722 confers redox regulation of v-Rel DNA-binding activity *in vitro* (24). However, since we failed to detect any direct redox effects

FIG. 3. (A) Inhibitory effects of p40-I κ B α on the DNA-binding activity of v-Rel and mutant 2722. 35 S-labeled v-Rel or 2722 were produced alone (lanes 1 and 4) or with p40 (lanes 2, 3, 5, and 6), incubated with 32 P- and BrdUrd-labeled κ B oligonucleotides, UV cross-linked, and immunoprecipitated with Ab1691 (lanes 1, 2, 4, and 5), or with anti-p40 antibodies (lanes 3 and 6). Arrow points to DNA-protein complexes. (B) Effect of excess unlabeled κ B oligonucleotides on p40-v-Rel and p40-2722 complexes. 35 S-labeled cotranslations of p40 and Rel were immunoprecipitated with anti-p40 antibodies before (lanes 1 and 3) or after (lanes 2 and 4) incubation with excess BrdUrd-labeled κ B oligonucleotides. Quantitation of 2722 coprecipitation with p40 was performed with the IMAGE-QUANT program on a PhosphorImager. (C) Inhibitory effect of p40 on transcriptional activity of c-Rel (CCR) and the c-Rel 2722 mutant. Average -fold inhibition of c-Rel- and c-Rel 2722-mediated CAT activation by p40 from three independent cotransfection assays in COS-7 cells is plotted.

on the *in vitro* interaction of v-Rel with p40 (data not shown), these results indicate that the DNA-binding region of 2722 in 2722-p40 complexes is more accessible to contact DNA than that of v-Rel complexed with p40. This would result in efficient DNA binding by 2722 in reaction mixtures contain-

ing p40 and in some presumably intermediate complexes containing p40 and 2722 cross-linked to DNA (Fig. 3A, lane 6).

A Peptide Derived from the DNA-Binding Region of v-Rel Specifically Reverts p40-Mediated Inhibition of DNA Binding. The results described above support the hypothesis that p40 may inhibit v-Rel DNA-binding by contacting residues in the vicinity of the conserved RXXRXXXC Rel DNA-binding motif, thereby competing with κ B DNA. To directly address whether p40 functionally contacts the DNA-binding motif of v-Rel, without introducing mutations that may alter Rel protein structure or affect its DNA-binding ability, we characterized the effects of Rel peptides on the DNA-binding activity of p40-v-Rel complexes. ³⁵S-labeled v-Rel produced alone or together with p40 was incubated with ³²P- and BrdUrd-labeled κ B oligonucleotides and increasing amounts of competing Rel peptides prior to UV cross-linking and immunoprecipitation with Ab1691. Peptide 1737 contains the basic residues of the v-Rel DNA-binding motif (EQPRQGR-TRFRY); peptide 1801 is derived from the unique C terminus of chicken c-Rel (CFYDTDGVTDE).

Reaction mixtures containing p40 and v-Rel bound DNA at 12% the efficiency of v-Rel alone (Fig. 4 *Left*). Addition of 30 μ g of competing peptide 1737 to p40-v-Rel binding reaction mixtures restored 65% of v-Rel DNA-binding activity (Fig. 4 *Left*). In sharp contrast, the nonspecific and mostly acidic competitor peptide 1801 had no significant effect on the binding activity of p40-v-Rel complexes (21% of v-Rel binding with 30 μ g of peptide; Fig. 4 *Left*). These experiments indicate that the basic competitor peptide 1737 relieves p40-mediated inhibition of v-Rel DNA binding. Furthermore, excess peptide 1737 did not affect v-Rel-DNA complexes in the absence of p40 (data not shown). Therefore, it appears that peptide 1737 may compete with the DNA-binding region of v-Rel for interaction with p40, allowing κ B DNA to contact the v-Rel DNA-binding motif. We propose that this interaction is important for p40-mediated inhibition of v-Rel DNA binding.

Covalent Modification of Peptide 1737 Interferes with Its Ability to Alleviate p40-Mediated Inhibition of v-Rel DNA Binding. To substantiate these findings, we analyzed the effects of chemically modified peptides in the competition assays. It was anticipated that covalent modification of arginine residues in peptide 1737 may compromise its ability to counteract p40-mediated inhibition of v-Rel DNA binding. Pretreatment of peptide 1737 with the arginine-specific modifying agent HPG (10 mM) (28, 29) significantly decreased its ability to alleviate the inhibitory effect of p40. Thirty micrograms of HPG-modified peptide 1737 restored 31% of v-Rel DNA-binding activity, equivalent to that seen with 30 μ g of

HPG-modified nonspecific competitor peptide 1801 (Fig. 4 *Right*). Combined with the results described above, these findings suggest that p40 inhibits DNA binding by contacting the v-Rel DNA-binding motif. Our data support the hypothesis of a mutually exclusive interaction between p40 and DNA with the RXXRXXXC DNA-binding region conserved in all Rel family proteins.

DISCUSSION

I κ B proteins play a central role in the intricate regulation of Rel protein function in the nucleus and in the cytoplasm. The conserved NLS region of Rel proteins was recently shown to interact with the I κ B α /MAD-3 inhibitor (8, 23). I κ B α /MAD-3 may thus preclude NLS recognition by nuclear transport proteins, resulting in the cytoplasmic retention of inactive I κ B-Rel complexes (8). Here we have examined the mechanism by which p40 may inhibit the DNA-binding and transcriptional activities of Rel proteins in the nucleus. Although this study does not rule out the possible interaction of p40 with other residues in the Rel homology region, our results demonstrate that the RXXRXXXC motif is required for p40-mediated inhibition of v-Rel DNA binding.

The details of the interaction between p40 and the DNA-binding motif of Rel remain to be clarified. However, several observations suggest that the arginine residues in the RXXRXXXC motif may form ionic interactions with the negatively charged C terminus of p40 (35% acidic residues), as proposed for the interaction of I κ B α /MAD-3 with the positively charged NLS region (8, 23). (i) The anionic detergent deoxycholate dissociates inactive I κ B α -Rel or I κ B α -NF- κ B complexes (30, 31), allowing DNA binding (13, 14). (ii) Alanine substitution of the arginine residues in the Rel DNA-binding region significantly decreased the association of v-Rel with p40 (Fig. 1B, lane 8). (iii) Anti-peptide antibodies raised against the DNA-binding region of v-Rel as well as antibodies raised against its N terminus recognized unbound v-Rel but failed to immunoprecipitate p40-v-Rel complexes (Fig. 2; refs. 30 and 31) or DNA-bound v-Rel (24). (iv) Peptide 1737 corresponding to the DNA-binding motif of v-Rel prevented p40 from inhibiting v-Rel DNA-binding (Fig. 4 *Left*). (v) Arginine modification of peptide 1737 with HPG compromised its ability to relieve p40 inhibition of Rel DNA binding (Fig. 4 *Right*).

The RXXRXXXC motif was previously shown to be essential for v-Rel DNA binding and to be functionally conserved among members of the Rel family (X. Xu and C.G., unpublished data; refs. 24 and 32). Yet, I κ B α interacts efficiently with v-Rel, c-Rel, and RelA but associates poorly with the p50 subunit of NF- κ B and fails to inhibit its DNA-

FIG. 4. Effect of Rel peptides on DNA-binding activity of p40-v-Rel complexes. The DNA-binding activity of p40-v-Rel relative to that of v-Rel is plotted as a function of the amount of unmodified (*Left*) or HPG-modified (*Right*) competing peptides added to DNA-binding reaction mixtures. Quantitation of [³²P]DNA present in DNA-protein adducts was performed with the IMAGE-QUANT program on a PhosphorImager. □ and ■, peptide 1737; △ and ▲, peptide 1801.

binding activity (8, 22, 23). It is therefore unclear whether the ability of p50 to escape negative regulation by I κ B α results from decreased interaction with the inhibitor or a different conformation of p50 that makes its DNA-binding region less accessible to I κ B α contact. It will be interesting to examine whether other I κ B proteins use a similar mechanism to inhibit the DNA-binding activity of other Rel family members.

Recent studies have shown that p40 differentially inhibits the DNA-binding activities of v- and c-Rel in extracts from chicken embryo fibroblasts coinfecting with Rel and p40 expression vectors (33). Whereas the DNA-binding activity of c-Rel was readily inhibited by p40, v-Rel retained 30–100% of its binding activity in cells expressing p40. The apparent discrepancy between the effects of p40 on the DNA-binding activity of v-Rel *in vitro* and *in vivo* may result from post-translational modification of v-Rel that may regulate its DNA-binding activity *in vivo*. The results reported by Diehl *et al.* (33) indicate that the central region of v-Rel may be the target for such modifications. Combined, these studies support the model that p40 and DNA compete for overlapping sequences in Rel proteins.

In contrast to the mutations in the RXXRXXXC motif, mutations in the NLS of v-Rel invariably abolished its association with p40, suggesting that this region constitutes the primary binding site for p40. Proper positioning of the proteins may allow further interaction of the RXXRXXXC region with p40, stabilizing the complex and blocking access to DNA. Excess competing peptide 1737 may disrupt p40 interaction with the RXXRXXXC motif, allowing DNA binding. Loss of the p40/RXXRXXXC contact may destabilize the p40–v-Rel complex, disrupting p40 interaction with the NLS. Alternatively, binding of DNA to the RXXRXXXC region may directly displace p40 from the NLS. Combined, the results described here demonstrate that inhibition of v-Rel DNA binding by p40 is mediated, at least in part, through its interaction with the RXXRXXXC motif. The nuclear localization of I κ B α inhibitors (17, 20, 21) suggests that I κ B α –Rel interactions may be critical for regulating the biological function of Rel family proteins.

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